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FLUNARIZINE ATTENUATES HYPOTHERMIA/REWARMING-INDUCED CHANGES IN PROTEIN AND WATER MOVEMENT ACROSS THE ENDOTHELIUM OF RATS

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Severe hypothermia damages endothelial cell cytoskeleton and significantly reduces tissue perfusion that is only partially restored on rewarming. The calcium channel blocker flunarizine (FL) was administered to examine the mechanisms and possible attenuation of hypothermia-induced changes in extravasation and perfusion. Blood and tissue samples were taken from 6 groups of 12 male rats: CN (control, normothermic). FLN (N rats given 1 mg/kg of FL by gavage), CHypo and FLHypo (cooled to and maintained at a Tc of 25oC for 1 hr following vehicle control or FL) and CRew and FLRew (rewarmed to a Tc of 35oC following Hypo). Recovery of Evans blue (Eb) bound albumin was used as a marker of protein extravasation. Because blood flow to most organs is significantly reduced during hypothermia, values for expected Eb were predicted from previously reported reduced flow and measured N extravasation, for Hypo and Rew tissues. In FLN rats, Eb was significantly ($p<0.05$) increased in liver (Li), intestine (I), kidney (K), and lung (Lu), suggesting that FL increased flow in these tissues. Hypo Li, I, Lu, muscle (M), and brain (B) and Rew Li exhibited greater than predicted Eb concentrations suggesting endothelial damage. In Rew tissues, FL increased Eb in Li, Lu, heart (H) and M (35.0 ± 8.0 vs. 28.3 ± 6.5, 47.9 ± 24.8 vs. 19.5 ± 9.1, 19.0 ± 6.3 vs. 13.4, 4.4 ± 1.8 vs. 3.0 ± 1.2, ug/gm dry wt of tissue, respectively) compared to CRew, with no difference in cooling or rewarming rates suggesting increased perfusion of these tissues. FL-induced increased extravasation was due to increased perfusion of rewarmed tissues thus potentially reducing ischemic damage resulting from hypothermia and rewarming.

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EXECUTIVE SUMMARY

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INTRODUCTION

While controlled mild to moderate hypothermia is commonly used during many surgical procedures to lower metabolic rate and reduce the dangers of ischemia during temporary diversion of the blood supply from target organs (2, 13), deep hypothermia can have serious pathophysiological consequences. In a clinical setting moderate hypothermia has reduced the severity of damage from traumatic brain injury in patients (21). Additionally, there is a renewed interest in using hypothermia for the clinical treatment of the edema and resulting increased intracranial pressure resulting from middle cerebral artery infarction (33, 36).

Rewarming from accidental severe hypothermia is associated with sudden, often fatal, vascular collapse characterized by falling cardiac output and sudden drops in peripheral resistance and BP (30, 39, 40). During severe hypothermia in rats there is a decrease in blood flow to brain, heart, liver, spleen, kidney, intestines, and muscle to 3-33% of normal values; even upon rewarming these flows may still be depressed to 5-55% of pre-hypothermic values (40). Cooled blood circulated through the brain has been reported to disrupt the blood brain barrier (28), and hypothermia altered endothelial cell structure (13). Cold exposure resulted in loss of transmembrane ion gradients and barrier function; ion pump failure resulted in increased Ca^{++} concentration and consequent cytoskeletal disruption followed by organ damage due to the breakdown of endothelial integrity (13). An increase in blood viscosity results in extravasation of plasma proteins and water (45); this loss of fluid from the vascular bed is postulated to be responsible for the diminished cardiac output (2) that may result in terminal vascular collapse.

Calcium acts as a regulator ion for many enzymes; the hypothermia-induced increase in Ca^{++} concentration may in itself be lethal, (44). Severe hypothermia results in increased intracellular Ca^{++} sequestration and consequent endothelial cytoskeletal disruption (13). Microvascular injuries induced by free radicals were prevented by pretreatment with the calcium channel blocker flunarizine (10) (FL, (E)-1-bis(4-fluorophenyl)methyl-4-(3-phenyl-2-propenyl) piperazine dihydrochloride). Below a core temperature (Tc) of 27° C there is an increase in blood viscosity (42), and increased viscosity increases extravasation. In a study by DeClerk et al. (7), FL attenuated the increase in blood viscosity following ischemia perhaps due to increased deformability of red blood cells and improved peripheral blood flow. Thus, the use of FL may not only attenuate extravasation due to endothelial gap formation and increased viscosity, but may also attenuate flow reductions characteristic of severe hypothermia.

Flunarizine is a selective calcium antagonist (27, 43) which readily crosses the blood-brain-barrier, protects cells from the effects of Ca^{++} overload without interfering with the normal cellular Ca^{++} homeostasis, and protects the endothelium by blocking the entry of excessive Ca^{++} into the cell (1, 5, 16). Additionally, FL does not alter normal blood-brain-barrier function with artificially induced hypertension (46).

A previously published study (22) established that there is a dose-response increase in endothelial permeability of rats subjected to increasing levels of hyperthermia. In a subsequent study (23), the calcium channel blocker flunarizine was used to attenuate this hyperthermia-induced extravasation. As the dose of flunarizine was increased from 0.3 to 3 mg/kg, there was a significant reduction of Evans blue (Eb) recovered from hyperthermic liver, kidney, lung, spleen, and duodenum. Endurance time in the heat to reach a Tc of 42.6°C was significantly increased with 1 mg/kg of FL compared to control and the reason for the increased endurance was speculated to be maintenance of vascular volume.

In earlier work Matthew et al. (24) examined the effects of a range of hypothermic core temperatures (Tc) on extravasation. Increased extravasation and the specific organ sites of this extravasation resulting from hypothermia to a core temperature (Tc) of 20, 25 and 30°C and rewarming from these Tc's were reported. Brain, heart, lung, liver, duodenum, spleen, kidney, and gastrocnemius tissues all exhibited significant differences from normothermic controls, and these differences were attributed to a combination of anesthesia, hypothermia-induced reductions in flow, and/or change in endothelial permeability.

Severe hypothermia damages endothelial cell cytoskeleton and significantly reduces tissue perfusion that is only partially restored on rewarming. The FL was administered to examine the mechanisms and possible attenuation of hypothermia-induced changes in extravasation and perfusion. Tveita (38) asserted that while the cause of cardiovascular collapse on rewarming was still unknown, it does involve alterations in the peripheral vascular bed and increased capillary leakage of plasma proteins. Recovery of Eb is commonly used to quantitate extravasation (15, 18, 26, 29, 35) or breach of the blood-brain barrier (4, 8, 34). Therefore, in this study, FL was evaluated for its ability to ameliorate hypothermia-induced extravasation and flow restriction by measuring changes in Eb extravasation and wet/dry weight changes in organ beds. Results from this study will increase knowledge of the pathophysiology of hypothermia and help to formulate pharmacological pretreatments to prevent such damage or treatments following the insult.

METHODS

ANIMALS

All experimental procedures were approved by our Institutional Animal Care and Use Committee and carried out with adherence to the "Guide for the Care and Use of Laboratory Animals," as revised in 1996 and to the U.S. Government Principles for Animal Use, 1985. A total of 76 adult male rats (Sprague-Dawley, Harlan, SD strain, 360-420 g) were used as follows: There were 6 groups (N=12/group) of experimental animals: CN (normothermic, controls), FLN (normothermic, 1 mg/kg FL suspended in 1:1 corn syrup in water, 4 ml/kg by gavage), CHypo (vehicle control, 4ml/kg 1:1 corn syrup in water by gavage, pentobarbital anesthesia, hypothermia, Tc 25 °C for 1 hr), FLHypo (FL, pentobarbital anesthesia, hypothermia), CRew (vehicle control,

pentobarbital anesthesia, rewarmed from hypothermia), and FLRew (1 mg/kg FL, pentobarbital anesthesia, rewarmed.) Four additional animals were used to obtain tissue blanks for the Eb assay. Data from anesthesia control groups for this study were previously published (24), and the relevance of that data on the results in this study is examined in the discussion. Animals were housed in individual wire bottom cages in the animal colony until the start of the study. Environmental conditions were maintained (26^{O}C, 50% relative humidity), lighting was controlled automatically (on, 0600-1800 h) and food (Purina Rodent Chow # 5001) and water were available *ad libitum* except during experimental intervals.

SURGICAL CANNULATION:

Animals were anesthetized (40mg/kg sodium pentobarbital, i.p.), given atropine (200 ug, i.m.) and antibiotics (Polyflex© ampicillin, 12.5 mg i.m.) prior to surgery. Through a mid-ventral neck incision, silastic® catheters were inserted aseptically into the external jugular vein for administration of Eb and blood withdrawal. All animals were allowed a 1-week recovery period prior to the study.

EXPERIMENTAL PROCEDURE:

A 0.5 ml blood sample was taken from the jugular catheter for determination of hematocrit (Hct) and % total protein concentration of the plasma (refractometer). Flunarizine (1 mg/kg, Sigma Chemical Co., St. Louis, MO; suspended in 4ml/kg, 1:1 corn syrup in water) or vehicle control (4ml/kg, 1:1 corn syrup in water) was administered by gavage. Thirty minutes later the rats were lightly anesthetized with sodium pentobarbital (Nembutal, 35 mg/kg, i.p.). Fifteen minutes later, a rectal probe was inserted 6cm beyond the anal sphincter and the animals were cooled as previously described (24). Each animal was placed in a cooling coil constructed of copper refrigeration tubing attached to a water bath initially set at 15^{O}C; over the next 15 min, temperature of the water bath was ramped down to 5^{O}C. Rats were cooled until they attained a rectal temperature (Tc) of 25^{O}C, where they were maintained for 1hr. Blood and tissue samples were collected from the Hypo groups after 1-hr at a Tc of 25^{O}C, and from the Rew groups after rewarming to a Tc of 35^{O}C by increasing the temperature of the circulating water to 37 ^{O}C over 20 min. Normothermic rats were neither given pentobarbital nor subjected to cooling, and tissues were sampled 2-3 hrs after administration of C or FL.

Following one hour of hypothermia for the Hypo groups or rewarming for the Rew groups, rats were removed from the coils and Eb (2.5mg/kg, 1:1 saline and rat serum to assure albumin binding, 2ml/kg) followed by 0.5 ml of saline flush was administered via the jugular catheter. After a further 15min, animals were deeply anesthetized with methoxyflurane; a blood sample taken by cardiac puncture; heart, liver, lung, intestine, gastrocnemius muscle, duodenal tissue, and brain were removed, washed twice in normal saline and ~0.5g portions placed in 3ml formamide overnight for Eb extraction. A second ~0.5g tissue aliquot was removed and dried to a constant weight to calculate wet-weight/dry-weight ratio.

EVANS BLUE ASSAY:

The following day, the supernatant was removed and filtered (Acrodisc syringe filter, 0.45 micron), and Eb concentration was read against a formamide blank (620nm, Perkin Elmer Lambda 2 dual-beam spectrophotometer). Because formamide extracts other substances that absorb light at 620nm, tissue blanks were obtained from normothermic rats without Eb injections. The mean absorbance of these tissues per gram of wet weight of tissue was subtracted from the absorbances of the appropriate experimental samples prior to calculating an Eb concentration from a standard curve generated for Eb in formamide. A final Eb concentration per gram of dry weight tissue was calculated.

STATISTICS:

All values are mean ± standard deviation (sd). For comparisons among groups, data were analyzed by one-way analysis of variance (Statistica© software) followed by Tukey's post hoc test for multiple comparisons. Student's "t" test was used for comparisons between actual and predicted values.

In order to determine how much extravasation is due to damaged endothelium or drug effect it is necessary to determine the change in extravasation that could be expected based solely on the change in organ blood flow during hypothermia and rewarming. An expected reduction in extravasation was predicted on the basis of the reduced flow as previously reported (24). Calculations of the predicted extravasation at 25 ^{o}C and after rewarming were derived from the values for change in blood flow to each organ reported by Tveita *et al.* (40) and are presented in Table 1.

RESULTS

Results of this study can be divided into three avenues of concern: 1) The effects of FL in normothermic tissue, 2) The effects of hypothermia and rewarming on extravasation of protein and tissue water content, and 3) The effects of FL on extravasation of protein and tissue water content in hypothermic and rewarmed tissues. Table 1 contains the Eb concentration in µg/g of dry weight (wt) of tissue for the normothermic control animals given no pentobarbital anesthesia (CN), the normothermic animals given 1 ml/kg of FL (FLN) and the values predicted for the hypothermic and rewarmed tissues based on a reduction in flow alone elicited by hypothermia and rewarming. The actual Eb concentrations measured in the hypothermic and rewarmed tissues with and without flunarizine indicative of albumin lost from the vasculature were compared to the values in Table 1 and are illustrated in Fig 1. Table 2 contains the wet/dry wt ratios of the tissues from the CN and FLN groups. Fig. 2 illustrates the wet/dry ratios of the hypothermic and rewarmed tissues and compares these to the control values in Table 2.

Table 1 Evans blue concentration in ug/g dry wt of tissue from normothermic and predicted values for hypothermic and rewarmed rats

Group	Liver	Intestine	Kidney	Lung	Heart	Muscle	Brain
CN[1]	20.6 ± 7.6	17.0 ± 3.6	16.7 ± 9.2	25.9 ± 13.7	19.0 ± 6.8	3.3 ± 1.1	1.7 ± 0.8
FLN[2]	28.4[c] ± 7.1	23.5[c] ± 6.5	23.7[c] ± 5.8	40.4[c] ± 13.1	22.4 ± 5.8	4.1 ± 1.6	0.7[c] ± 0.6
Pred hypo25[3]	12.2 ± 4.5	9.2 ± 1.9	8.7 ± 4.8	14.3 ± 7.5	10.3 ± 3.7	2.3 ± 0.8	0.9 ± 0.4
Pred rew25[4]	10.9 ± 4.0	11.4 ± 2.4	9.7 ± 5.3	17.1± 9.0	13.1 ± 4.7	2.4 ± 0.8	1.1 ± 0.5

1 Normothermic control without nembutal anesthesia.
2 Flunarizine 1 mg/kg in normothermic animals.
3 Values predicted for hypothermic tissue.
4 Values predicted for rewarmed tissue.
C Significantly ($p<0.05$) different from CN.

Table 2 The wet weight /dry weight ratio of tissues from normothermic rats with and without 1 mg/kg flunarizine.

Group	Liver	Intestine	Kidney	Lung	Heart	Muscle	Brain
CN[1]	3.21 ± 0.09	4.22 ± 0.13	3.91 ± 0.17	4.82 ± 0.11	4.54 ± 0.07	4.15 ± 0.06	4.46 ± 0.13
FLN[2]	3.19 ± 0.04	4.30 ± 0.15	3.89 ± 0.12	4.99[c]± 0.12	4.49 ± 0.07	4.07 ± 0.05	4.25[c]± 0.12

1 Normothermic control without nembutal anesthesia.
2 1 mg/kg flunarizine in normothermic animals.
3 C Significantly ($p<0.05$) different from CN.

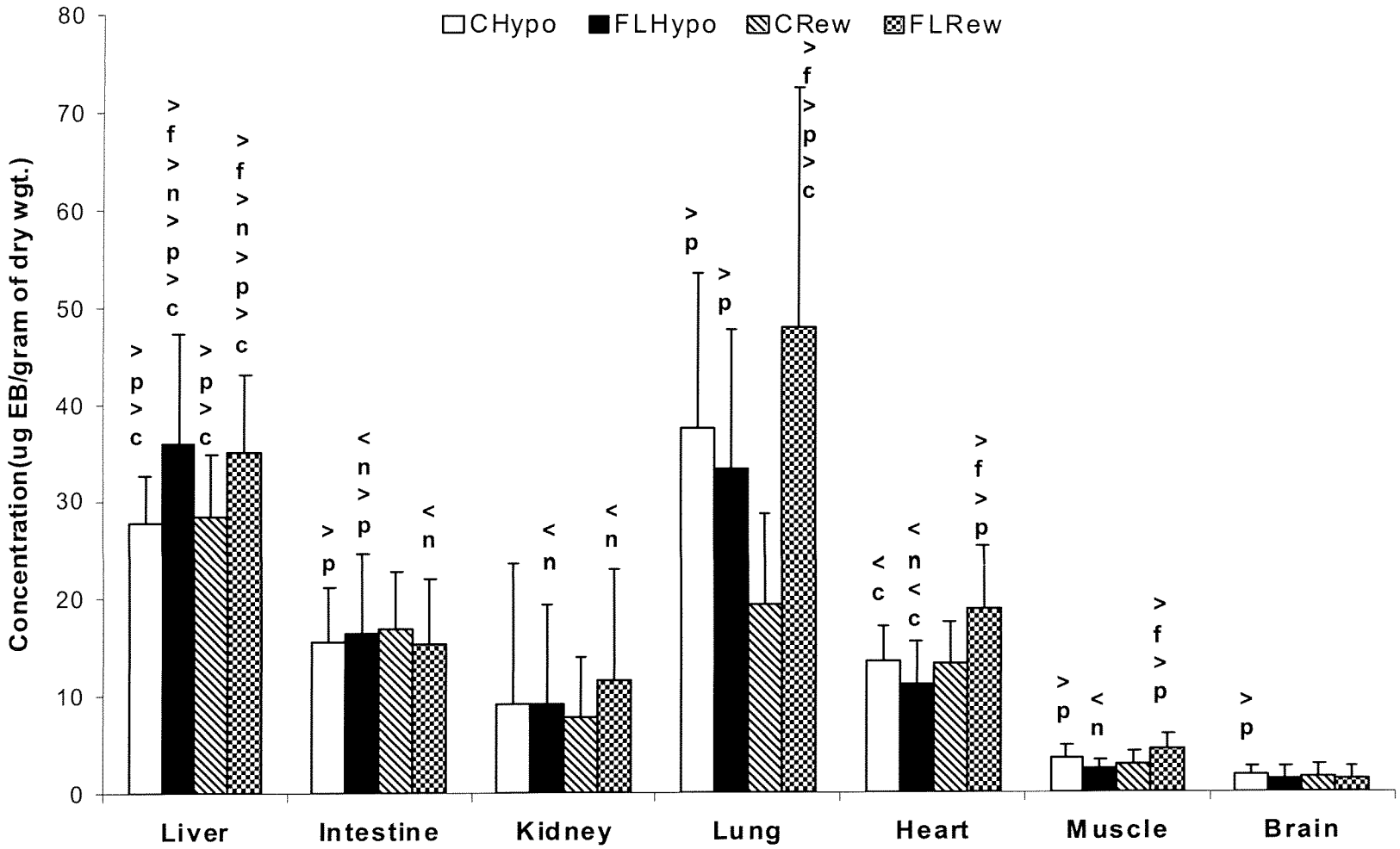

Figure 1 Concentration of Evans blue for each of the tissues for hypothermic groups with and without FL and the rewarmed groups with and without FL. Values are mean ± standard deviation. Values that are significantly ($p < 0.05$) different are represented as follows: whether the values is greater than (>) or less than (<) is indicated above the letter; the letters represent- 'c' than normothermic control values (Table 1), 'p' than predicted values (Table 1), 'n' than normothermic FL group (Table 1), 'f' FL value is significantly different from corresponding group not receiving FL.

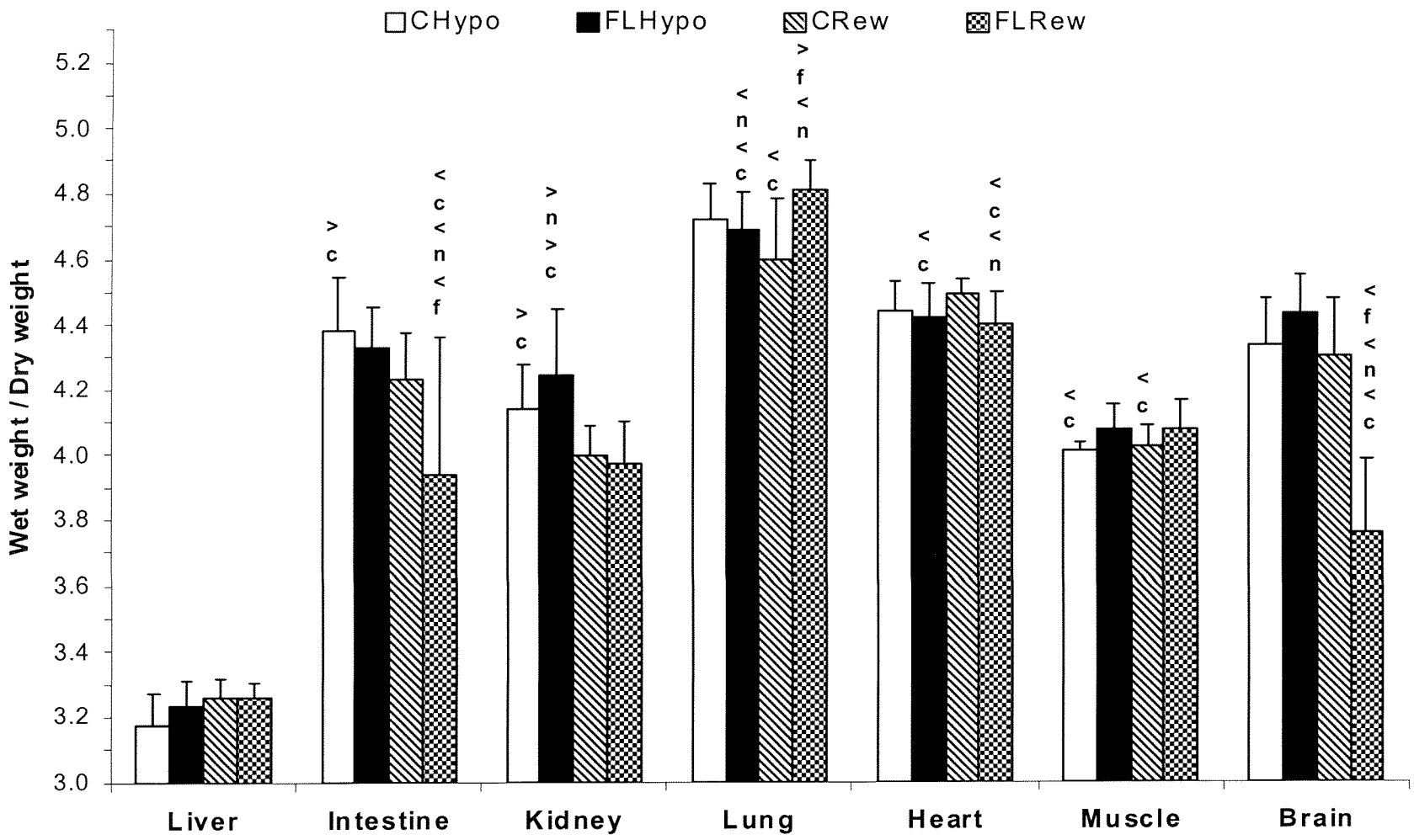

Figure 2 Wet weight/ dry weight ratio for each of the tissues for hypothermic groups with and without FL and the rewarmed groups with and without FL. Values are mean ± standard deviation. Values that are significantly ($p < 0.05$) different are represented as follows: whether the values is greater than (>) or less than (<) is indicated above the letter; the letters represent- 'c' than normothermic control values (Table 2), 'n' than normothermic FL group (Table 1), and 'f' FL value is significantly different from corresponding group not receiving FL.

FL IN NORMOTHERMIC TISSUE:

Administration of FL to normothermic rats (Table 1) resulted in a significantly ($p<0.05$) increased concentration of Eb-bound albumin in intestinal (38%), renal (42%), pulmonary (56%), and hepatic (38%) tissues as well as a significant decrease in the brain (58%). A comparison of the wet/dry wt ratios of the FLN to the CN group (Table 2) indicated that FL increased water content of the lungs and decreased water content of the brain. Therefore, FL increased extravasation of protein in the intestine, kidney, lung, and liver as well as decreasing it in the brain; FL increased lung tissue water content and decreased that of brain. Only those tissues with a change greater than 50% in protein content exhibited a gravimetrically measurable corresponding change in water content.

PROTEIN EXTRAVASATION AND WATER CONTENT OF HYPOTHERMIC AND REWARMED TISSUE:

Using the predicted values for extravasation of albumin-bound Eb from Table 1, the measured values for each hypothermic and rewarmed tissue were compared to the values that might be expected if hypothermia and rewarming-induced changes in flow were the only factors responsible for a change in protein extravasation. In both hypothermic (CHypo) and rewarmed (CRew) hepatic tissue (Fig. 1), extravasation of albumin-bound Eb was greater than predicted and greater than CN. In intestinal, pulmonary, gastrocnemius muscle, and brain tissue, the hypothermic tissue contained significantly higher concentrations of Eb than predicted, but the rewarmed tissue contained the predicted concentration. In renal and cardiac tissues both hypothermic and rewarmed tissues contained the predicted concentrations of Eb. Wet wt/dry wt ratios (Fig. 2) for hypothermic intestinal and renal tissue were significantly greater than for CN while hypothermic muscle and rewarmed pulmonary and muscle tissue had significantly lower ratios than CN. Therefore, hypothermic liver, intestine, lung, muscle and brain all exhibited increased leakage of protein while only liver continued to exhibit increased extravasation in rewarmed groups. Edema was evident only in hypothermic intestinal and renal tissue, but dehydration of rewarmed tissue was evident in lung and muscle tissue.

FL EFFECTS IN HYPOTHERMIA AND REWARMING:

Values illustrated in Fig. 1 and Fig. 2 were compared to determine the impact of FL administration on Eb content and wet wt/dry wt ratios of hypothermic and rewarmed tissues. In both hypothermic and rewarmed hepatic tissue, FL administration increased Eb content (Fig. 1) above that induced by hypothermia or FL alone (Table 1); therefore, in hepatic tissue the effects of FL and hypothermia on Eb content were additive. There were no differences in hepatic water content among any of the experimental groups. FL administration did not alter Eb content of intestinal or renal tissue in either hypothermic of rewarmed groups. Wet wt /dry wt ratios were not changed in renal tissue, but FL significantly reduced water content of rewarmed intestine. In pulmonary and cardiac

tissue, FL did not change Eb extravasation in hypothermic tissue; however, FL significantly increased extravasation in rewarmed pulmonary (145%) and cardiac (42%) tissue. Wet wt/dry wt ratios of pulmonary but not cardiac tissues was increased similar to the phenomenon noted in normothermic tissue where a greater than 50% increase in protein extravasation was required to be reflected in increased water content. In muscle and brain tissue where hypothermia alone induced an increase in Eb extravasation compared to the predicted value, FLHypo groups had Eb contents that were not greater than predicted. FL increased Eb extravasation in rewarmed muscle but not in rewarmed brain. FL increased wet wt/dry wt ratio of muscle tissue in hypothermic and rewarmed groups so that it was no longer less than that of normothermic controls. FL dramatically decreased rewarmed brain water content but did not alter hypothermic water content.

The increase in extravasation (Table 1) resulting from the FL-induced vasodilatation and accompanying increase in flow was not accompanied by a change in either cooling rate (CHypo, –0.124 ± 0.044 vs. FLHypo, –0.132 ± 0.039; CRew, –0.143 ± 0.030 vs. FLRew, –0.136 ± 0.036 °C/min) or rewarming rate (CRew, 0.190 ± 0.059 vs. FLRew, 0.190 ± 0.030 °C/min) of the FL-treated animals.

Table 3 Hematocrit and % total plasma protein concentration

	Pre-HCT	Post-HCT	Change pre to post HCT	Pre % TPP	Post % TPP	Change pre to post TPP
CN	49.6 ± 1.9	44.3[a] ± 1.8	-5.1 ± 2.2	7.1 ± 0.3	5.8[a] ± 0.3	-1.2 ± 0.3
FLN	49.5 ± 2.3	43.9 ± 1.5	-6.0 ± 2.0	7.0 ± 0.2	5.7 ± 0.3	-1.3 ± 0.3
CHypo	50.0 ± 1.8	46.1 ± 2.0	-4.0 ± 1.8	7.1 ± 0.3	5.9 ± 0.4	-1.2 ± 0.4
CREW	48.7 ±1.2	43.9[b] ± 2.0	-4.8 ± 1.8	7.1 ± 0.3	5.4[b] ± 0.3	1.7 ± 0.2
FLHypo	48.8 ±1.9	45.2 ± 2.2	-3.5 ± 1.5	7.2 ± 0.2	5.9 ± 0.4	-1.4 ± 0.4
FLRew	50.2 ± 2.1	42.6[b] ± 2.1	7.3[bc] ± 1.2	7.0 ± 0.2	5.3[b] ± 0.1	-1.7 ± 0.2

All values are mean % ± SD.
a All post-samples are significantly ($p<0.001$) different from pre-samples.
b Rewarmed significantly ($p<0.02$) different from hypothermic.
c FLRew significantly ($p<0.001$) different from CRew.

Δ IN HCT AND % PLASMA PROTEIN:

Table 3 contains the Hct and % total plasma protein (TPP) concentration determined prior to the start of each experiment (pre) and again from the large blood

sample taken during exsanguinations via cardiac puncture prior to tissue harvest (post). The mean post Hct and % TPP values of all groups were significantly ($p<0.001$) lower than pre values. The Rew Hct and % TPP values were significantly ($p<0.02$) lower than the corresponding values for the Hypo groups for both C and FL. Additionally, the FLRew group had a significantly ($p<0.001$) lower Hct than the CRew group.

DISCUSSION

The results and conclusions from this study are based on the measurement of Eb extravasation and wet/dry weight ratio of tissues. The measured Eb is an index of albumin loss from the vasculature. The wet wt/dry wt ratio is an index of the change in net tissue water content as a result of loss of water from the tissues both via dehydration and diuresis as well as the gain of water by the tissues resulting from edema.

Flunarizine is a calcium channel blocker; properties of this class of drugs would be expected to impact physiology during hypothermia and rewarming. A general characteristic of calcium channel blockers is that of vasodilatation. Vasodilatation of superficial peripheral vasculature could be a detriment in a cold environment resulting in an increased rate of heat loss to the environment. However, in this study the administration of FL did not change the cooling rate consistent with the work of Centonze et al., (3) on Raynaud's syndrome. Some calcium channel blockers have shown promise in the treatment of the extreme vascular constriction characteristic of Raynaud's syndrome, however FL pretreatment did not relieve this cold-induced constriction in the hands; FL did not dilate surface vasculature. In addition to vasodilatory and anti-vasocontrictive effects that differ in different organs, calcium channel blockers have been shown to have direct antiischemic and cytoprotectant effects and these protective effects also differ in different organs (41).

In normothermic tissues, FL increased extravasation of protein in the intestine, kidney, lung, and liver and decreased it in the brain; FL increased lung tissue water content and decreased that of brain. As FL is a known vasodilator in normothermic tissues, the increases in extravasation were most likely due to increased FL-induced flow in these tissues. Hypothermic liver, intestine, lung, muscle and brain all exhibited increased leakage of protein while only liver continued to exhibit increased extravasation in rewarmed groups. As hypothermia decreased organ blood flow and disrupted the endothelial cytoskeleton, the increased extravasation most likely indicated cytoskeletal damage. Increased Eb extravasation indicated that FL administration significantly increased perfusion in rewarmed liver, lung, heart, and muscle tissue but of the hypothermic tissues perfusion was increased only in the liver.

An increase in blood flow increases the capillary diffusion capacity of a vessel, because the concentration gradient is maintained for a greater time during the transit. This is a particularly important consideration in skeletal muscle and brain as well as heart and adipose tissue where permeability is limited (6). Water diffuses quickly using all of the capillary surface both cellular and paracellular resulting in rates of water diffusion that are 10-100 times greater than the rate of other solutes that are restricted

to intercellular clefts. This may help to explain the dramatic decrease in brain water content on rewarming.

An increase in intestinal blood flow increases hepatic blood flow as 70-80% of hepatic flow is from the gastrointestinal venous outflow. Thus in this study, the increases in Eb seen in hypothermic and rewarmed liver with FL may simply reflect result of vasodilation and increased flow in the intestine. The FL-induced changes in flow resulted in the greatest changes in rewarmed liver and lung, which would be expected as they are both reticuloendothelial system tissues with facilitated trans-endothelial transport.

Another potential mechanism by which FL could be beneficial is that of antioxidant. We have previously demonstrated organ-specific, dose-response vasogenic edema (extravasation) resulting from hyperthermia (22), from hypothermia and from rewarming following hypothermia (24). Hyperthermia alters the structure of endothelial cells changing their shape and reducing the integrity of the endothelial vascular barrier (17) resulting in increased vascular permeability and extravasation. Local heating of the pial vasculature of rats resulted in platelet aggregation, local microvascular occlusion (11), and ischemia, all of which were induced by free radicals and attenuated by the calcium channel blocker FL (10). Flunarizine attenuated the hyperthermia-induced extravasation and the mechanisms of its action appeared to be that of an antioxidant as well as a calcium channel blocker (23).

A number of studies have directly linked hypothermic injury to the production of free radicals. Increased antioxidant defense is one of the physiological adaptations found to protect the brain against a variety of insults in hibernating animals (9). In a study of cultured rat hepatocytes and liver endothelial cells incubated in a cold culture medium down to 4°C, cold-induced release of oxygen free radicals were the main cause of cellular damage (31). Pretreatment of the cell cultures with α-tocopherol (vitamin E) prevented the injury. Lipid peroxidation was induced in rat brain, liver, skeletal muscles and heart muscle during the induction of hypothermia to 30°C, and on reaching 20°C was 30-160% greater than during normothermia (20).

As Tc decreases, fluid shifts out of the vasculature, which increases Hct and results in increased viscosity and compromised circulation. Diuresis early in hypothermia reduces total vascular volume but at least temporarily increases kidney water content in hypo animals as seen in Fig 2. Diuresis early in the course of hypothermia is probably a reflex response to increased blood pressure secondary to peripheral vasoconstriction. Additionally, there may be osmotic movement of fluid into cells as a result of hypothermia-induced metabolic activity. This intracellular movement of water may be reversed as cooling progresses and reflexes are abolished resulting in increased extracellular water or edema. During rewarming, blood volume has however been shown to increase and may actually exceed pre-hypothermic levels (14)

In the present work, only those tissues with a greater than 50% change in protein content exhibited gravimetrically measurable corresponding changes in water content. Lack of a change in liver water content (Fig. 2) may be due to the lower wet/dry wt ratio

of liver tissue in general due to the high lipid content making the tissue more hydrophobic. Alternatively, in liver tissue the % changes in protein, while significant, did not reach the 50% level necessary to be reflected in a change in wet/dry wt ratio.

In Table 3, the significant decrease in Hct and protein content of blood from the small-volume pre to the large-volume post sample in all groups is due to the rapid movement of fluid from the interstitium into the vasculature during exsanguination via cardiac puncture used to obtain the sample. This large sample was taken in order to be as consistent as possible in removing as much blood as possible from tissues so that the Eb extracted would represent that in the tissues and to minimize the Eb from residual blood in the vasculature. As previously reported (14), water entered the vasculature during rewarming as evidenced by the significantly greater decrease in Hct and %TPP content of the plasma from pre to post in the Rew groups than in the Hypo groups with and without FL. Additionally, FL appears to have further increased the plasma content of the blood over that seen in rewarmed control animals. Thus, providing a greater circulating volume and better potential perfusion of rewarmed tissues.

Anesthesia control groups were included in this study but the data are not presented in this report, because they were previously published (24). Briefly, three groups of normothermic rats were given the same dose (35 mg/kg, i.p.) of pentobarbital anesthesia as the hypothermic and rewarmed groups whose data are presented. Blood and tissue samples were taken from the three groups at 2, 3, and 4 hr following pentobarbital administration respectively. In the present study, tissues were harvested from the hypothermic and the rewarmed groups 166 ± 46 min and 198 ± 24 min respectively, after pentobarbital administration. Therefore, data from the hypothermic groups would have fallen between the results from the 2 and 3-hour anesthesia groups, and data from the rewarmed groups would have fallen between the results from the 3 and 4 hour anesthesia groups. The wet/dry wt ratios in the anesthesia groups indicated that at 2 hrs there was a significant increase in water content of lung kidney and intestine that was resolved by 3 hours which may explain the increase in water content of these tissues in hypothermic but not in rewarmed groups. Eb content of tissues in the anesthesia groups indicated that there was less protein extravasation from brain, muscle, lung and kidney at 2 hours and continued to be decreased only for muscle and brain at 3 hours. These differences are opposite to those changes noted in hypothermic and rewarmed groups; therefore, the differences in protein extravasation seen in hypothermic and rewarmed groups are due to either the hypothermia and rewarming or the FL treatment and are not related to anesthesia administration.

The endothelial tissues in each of the organs examined have unique structural and functional properties which serve to help explain some of the differences seen with hypothermia or rewarming with and without FL in this study. Table 4 summarizes some of these differences (12, 19, 37). Structurally, liver tissue has a discontinuous endothelium, no basement membrane and is readily permeable to proteins. The intestine and kidney are similar in that they have fenestrated endothelia, continuous

basement membranes and protein permeability that are limited but increase with ischemia. Lung and heart both have continuous endothelia and basement membranes, but the lungs have a high degree of vesicular protein transport. Muscle and brain also both have continuous endothelia and basement membranes, but muscle does have specialized pores between endothelial cells and vesicular transport of proteins, where as the brain under normothermic conditions has minimal vesicular transport. (37)

Table 4. Endothelial characteristics of each tissue type

	Liver	Intestine	Kidney	Lung	Heart	Muscle	Brain
Endothelium type	dis-continuous	fenestrated	fenestrated	continuous	continuous	continuous	continuous
Basement membrane	none	continuous	continuous	continuous	continuous	continuous	continuous
Protein transport	Readily permeable	Increases with ischemia	Increases with ischemia	High vesicular transport		Pores between cells, vesicles	Minimal vesicle transport

Most hydrophilic molecules such as albumin must cross the endothelial barrier of the capillaries outside of the cells (i.e. through pores between cells) except for water that may also go through the cells (6). Lipid soluble molecules can diffuse through lipid membrane components, but water-soluble molecules can only cross the endothelial cells via pore in junctions between cells. Different tissue types have different junction with the brain having the tightest junctions allowing passage of the fewest and smallest water-soluble molecules of any tissue. For most capillary beds the composition of interstitial fluid and plasma is the same except for protein content, but the blood-brain barrier is so impermeable that differences in concentration of many solutes determine fluid flow (25).

Endothelium in hepatic tissue has fenestrations that allow easy exchange and equilibration between plasma and interstitial spaces. This ready exchange may be the reason for the lack of change in hepatic wet/dry wt ratio in any of the groups. Sympathetic stimulation suppresses autocontrol of hepatic blood flow and results in shunting of hepatic flow to the general circulation. However, the increase in Eb in CHypo and CRew over predicted and over that in CN indicates increased extravasation; further increase in Eb with FL indicated increased flow resulting from vasodilatory effects of FL.

Gastro-intestinal tissue is highly responsive to sympathetic stimulation resulting in significant vasoconstriction and the shunting of blood from the GI tract to other tissues particularly during hyperthermia, hypothermia and exercise. This may result in

prolonged ischemia and resultant tissue damage (32). In the intestine, capillary blood pressure is low and plasma colloid osmotic pressure is usually higher thus facilitating absorption of water into the vasculature. Thus increased flow resulting from FL-induced vasodilation evident in FLN (Table 1) could have attenuated the normal hypothermia and rewarming induced vasoconstriction. This increased flow (19) could result in more water removed from intestinal tissue as seen in FLRew.

While the lung, brain and muscle all have continuous endothelia and basement membranes, the mechanisms of protein transport across the endothelium differ. The lung has extensive vesicle exchange; the muscle has larger pores between endothelial cells particularly in the post capillary venules; but the brain has minimal protein transport by either mechanism (32). This could explain why FL administration in both the lung and muscle resulted in increased protein extravasation while increased extravasation was not seen in the brain.

Like the gastrointestinal tract, skeletal muscle is also sensitive to sympathetic stimulation that can increase arterial resistance and decrease flow resulting in shunting of flow to other areas during hyperthermia and hypothermia, but exercise greatly increases flow (32). Although muscle contains continuous endothelium and a basement membrane, pharmacological agents have been shown to affect the permeability to plasma protein (37). Thus the increase in flow resulting from FL administration in rewarmed muscle would be expected to increase protein extravasation as seen with the increase in Eb concentration in Fig.1.

The blood-brain barrier is the endothelium of the brain capillaries. The tight junctions between endothelial cells contain no fenestra and very few pinocytotic vesicles. The structure is similar to an epithelial membrane. Transport of inorganic ions is much slower across the blood-brain barrier than in other capillaries and Ca^{++} transport is slower than Na^{+}, K^{+}, and Cl^{-} transport. Movement of water and tracers across the blood-brain barrier is strongly dependent on changes in blood flow (12). Evans blue-bound albumin does not cross the intact blood-brain barrier. Since protein content of brain tissue did not change with rewarming while water content dropped significantly, the difference across the membrane responsible for the movement of water out of the tissues could have been the result of a large increase in other osmotic entities such as inorganic ions in the blood flowing more rapidly through the brain vasculature on rewarming with FL.

During hypothermia, Tveita et al (40) determined that the reduced cardiac output was due to a reduced heart rate, but during rewarming cardiac output returned only to 33% of control due to a reduced stroke volume. Skin and muscle blood flows on rewarming were not significantly different from control values despite the fact that cardiac output was only 33% of control values; thus, blood flow distribution on rewarming would seem to be detrimental to survival. A contributing factor in the reduced blood flow was an increase in vascular resistance due to aggregation of red blood cells and hemoconcentration. Interstitial and plasma colloid osmotic pressure measurements indicated that there was a significant decrease in the plasma and an

increase in the interstitial on rewarming. This would seem to indicate a significant increase protein extravasation consistent with the greater than predicted extravasation of albumin-bound Eb in the intestine, lung, muscle, liver and brain tissue found in the present study (Fig. 1). Flunarizine administration increased the flow (as evidenced by increased protein extravasation) in heart, liver, lung and muscle without reducing flow to any organ bed; thus, providing more beneficial perfusion and potentially increasing survival.

Flunarizine pretreatment appears to have been beneficial in these studies of hypothermia and rewarming as well as the previously reported studies (23) of hyperthermia. All of the tissues that exhibited an increase in extravasation in the FLN group (liver, intestine, kidney, and lung, Table 1) were the same tissues in which hyperthermia-induced extravasation was reduced by FL in the hyperthermic rats. This may indicate that these tissues are the most sensitive to FL effects.

CONCLUSIONS

In conclusion, although others have previously measured hemodynamics in hypothermia and rewarming, this paper is the first to report changes in organ water content and protein extravasation. In our previous work in hyperthermia, FL decreased endothelial cell deformation thus decreasing gap formation between cells and consequent excessive hyperthermia-induced extravasation. In hypothermia and rewarming, FL-induced vasodilation and increased red blood cell deformability thereby improving perfusion to tissues and reducing potential ischemia. Thus, the calcium channel blocker FL has different effects in hypothermia and hyperthermia, but beneficial effects in both cases.

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